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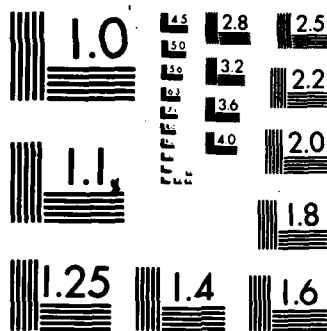
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Liposomal-Encapsulated Stroma-Free
Hemoglobin as a Potential Blood Substitute

Annual Progress Report

by

C. Anthony Hunt, Ph.D.

January 2, 1980
(For the period April 1979 through March 1980)

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Summary

We have successfully applied a new technique of high-yield encapsulation for liposome encapsulation of stroma-free hemoglobin (SFH). The procedure results in encapsulation of approximately 20% of a given SFH solution. The resulting hemoglobin-containing liposomes (HCL) range in size from approximately 0.05 to 1.0 μ in diameter. There is little or no binding of hemoglobin to these liposomes. The oxygen binding properties of the HCL are equal to or better than a corresponding amount of SFH in buffer. At 20° or 4°C leakage of hemoglobin from the HCL is minimal over 72 hrs. The HCL can be easily centrifuged and resuspended without significant lysis. Mild sucrose gradients (3-6%) can be used to separate the denser, hemoglobin-rich liposomes, from the lighter, hemoglobin-poor liposomes. These HCL are less sensitive to osmotic shock than are RBC's.

In vivo studies show that uptake of liposomes by the reticuloendothelial system (RES) is not the primary mechanism for blood clearance of liposomes; disposition of intravenously administered liposomes is the result of circulating instability, tissue binding and, finally, RES uptake. Further, each of these processes is saturable. Results show that the liposomes of interest can be made reproducibly. Preliminary data suggest that the circulating half-life of liposomes can be dramatically increased by changes in composition, such as substitution of sphingomyelin for the phosphatidylcholine.

Results of these initial studies are encouraging. Hemoglobin containing liposomes should be evaluated further as a potential blood substitute.

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Annual Report to the Acquisition Group, U.S. Army
Medical Research and Development Command

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Liposome-Encapsulated Stroma-Free
Hemoglobin as a Potential Blood Substitute
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Annual Progress Report
to the
Acquisition Group
U.S. Army Research and Development Command

Description of Project

The objective of this project has been to develop procedures to encapsulate stroma-free hemoglobin (SFH) in liposomes (biodegradable phospholipid vesicles) such that the resulting stability, O₂ binding properties, and in vivo properties are superior to those of SFH as an effective, safe blood substitute.

Technical Objectives

1. Identify the procedure which gives the highest efficiency for encapsulating SFH.
2. Determine which procedure is both least traumatic and most rapid for separating the unencapsulated SFH from the hemoglobin-containing liposomes.
3. Establish if the O₂ binding properties of hemoglobin, Hgb, encapsulated in liposomes is different from simple solutions of Hgb.
4. Quantify the extent to which Hgb binds to liposomes.
5. Determine the dose level at which the in vivo disposition of HCL becomes dose-dependent and if at the higher doses the circulating life-time of liposomes is extended.
6. Demonstrate that the extrusion techniques to produce liposomes is reproducible and if the size-distribution of the larger liposomes (0.5-1.0μ) can be improved.

Abbreviations Used

HCL: hemoglobin-containing liposomes	PC: Phosphatidylcholine
SFH: stroma-free hemoglobin	PA: phosphatidic acid
Hgb: hemoglobin	CH: cholesterol
RES: reticuloendothelial system	α-T: α-tocopherol
FCE: fluorocarbon emulsion	

Background

In our original proposal we defined liposomes, summarized the literature relevant to our contention that hemoglobin-containing liposomes, HCL, suspended in a solution of SFH may be superior to SFH as a militarily useful blood substitute, and outlined our data on the development and evaluation of liposomes for in vivo use. At that time the idea seemed promising, but sufficient data was not available to make initial judgments. Our proposal was designed as a feasibility study to begin collecting such data.

For this idea to prove viable a series of important questions must be answered affirmatively:

1. Can new procedures be developed which will allow encapsulation of sufficient amounts of SFH?
2. Will liposome encapsulation of SFH avoid adverse effects on the O₂ binding properties of SFH?
3. Will the HCL be reasonably stable?
4. Can the rapid clearance of liposomes, apparently by the RES, be retarded such that the circulation half-life of HCL is greater than that of SFH?
5. Will the HCL suspensions be less toxic than SFH solution?

Over the past 9 months we have focused primarily on questions #1, 3 and 4, and less on questions 2 and 5.

Results

Preparations of HCL, Hemoglobin-Containing Liposomes - Although a variety of procedures have been described to prepare liposomes (1), only three are practical on a large scale and, thus, we have focused on these: mechanical dispersion of dried phospholipids(2), sonication(2) and emulsion evaporation(3).

Mechanically dispersed liposomes are multilamellar and initially heterogeneous in size with aqueous layers between concentric lipid bilayers. They result from hydration and subsequent mechanical dispersion of dried lipids in an aqueous solute solution. Typically, PC/PA/CH/ α -T (in the molar ratio 4/1/5/.1) liposomes prepared in this manner encapsulate 3-5 μ l of aqueous solution for each mg (total) of lipid (in this case 1mg = 1.73 μ M) and the encapsulated concentration of small solute molecules will equal that in the original aqueous solution when no liposome-solute binding occurs; however, large molecules such as albumin and Hgb are somewhat excluded during the encapsulation process. For example, when a liposome dispersion containing 10mg/ml of the above lipid mix is prepared in the presence of ¹⁴C-sucrose, 5% of the aqueous solution is encapsulated; however, under identical conditions using a mixture of Hgb and sucrose only 0.5% of the Hgb is encapsulated while 5% of the sucrose is encapsulated (Table 1). Apparently Hgb is excluded during liposome formation resulting in a Hgb concentration inside the liposome only 10% that in the original solution. We conclude that this procedure, under our conditions, is not suitable for HCL formation.

Sonication gives even poorer results (Table 1). Sonicated liposomes are generally 0.02-0.05 μ m in diameter and are prepared by extended sonication of mechanically dispersed liposomes. In some case sonication improves solute capture, but not with Hgb. Sonicated liposomes of the above composition encapsulate about 1 μ l of aqueous solution (based on ¹⁴C-sucrose) for each mg (total) of lipid. When such sonicated liposomes are prepared at 10 mg/ml in Hgb/¹⁴C-sucrose they typically encapsulate 1% of the sucrose, but only 0.2% of the Hgb. Additional problems with sonication are that lipid oxidation and protein precipitation can occur.

Emulsion evaporation is the best procedure for preparation of HCL (Table 1). Our procedure in its current stage of development consists of preparation of an emulsion of aqueous SFH in ether (a water in oil emulsion), evaporation of the

organic solvent to form a gel and subsequent phase inversion leading to liposome formation (Results are seen in Fig. 1a). Specifically:

1. The lipids which comprise the HCL membrane (in this report PC/PA/CH/ α -T in the molar ratio 4/1/5/0.1) are dissolved in proxide-free (imperative) diethyl ether.

2. The ether solution is mixed with an appropriate volume of aqueous SFH and processed (shaken) to form an emulsion.

NOTE: The success of the procedure depends on a proper balance between the amount of lipid, the volume and type of organic phase, the volume of the aqueous phase, and the amount of Hgb. The emulsion must be stable--without significant phase separation--for at least 30 min. We have evaluated a variety of the above variables and have observed that for a 7% SFH solution and the above lipid mix, ether is the optimum solvent, 200 μ M of lipid are needed per ml of aqueous solution and the best ether-to-aqueous volume ratio is 4:1.

3. The ether phase of the emulsion is removed under vacuum to form a stable gel. At this point all of the Hgb is entrapped within a lipid bilayer network.

4. A 10-25% excess of aqueous phase is added. Vigorous shaking breaks the gel giving rise to liposomes.

5. Unencapsulated Hgb is removed by dialysis or centrifugation. We have found that when the ether-aqueous ratio is 4:1, 200 μ M/ml (aqueous) of the above lipid mix is optimum for emulsion formation when the aqueous phase is 7% SFH. Under these conditions the HCL encapsulate at least 20% of the original Hgb. See Figure 1A&B.

Table 1

Hgb Encapsulation in Liposomes Prepared Using Different Procedures.

<u>Procedure</u>	<u>μl of Hgb Solution Encapsulated per μM Lipid^a</u>	
	<u>Based on Measures of Trapped Sucrose^b</u>	<u>Based on Measures of Trapped Hgb^c</u>
Mechanical Dispersion	2.75	0.28
Sonication	0.55	0.11
Emulsion Evaporation	1.00	1.10(2.60) ^d

a Solutions of SFH, 7%, at pH 7.4 were used as the initial solution; lipid composition in each case was PC/PA/CH/ α -T in the molar ratio of 4/1/5/.1.

b The SFH solution contained 10mM ¹⁴C-sucrose.

c Encapsulated Hgb was measured spectrophotometrically after extraction of lipids.

d When it is assumed that O₂ saturated Hgb binds 1.34 ml O₂ per 1g Hgb, the amount of encapsulated Hgb could be calculated from the O₂ binding data.

FIGURE 1A. Primary steps for the formation of HCL by Emulsion Evaporation.

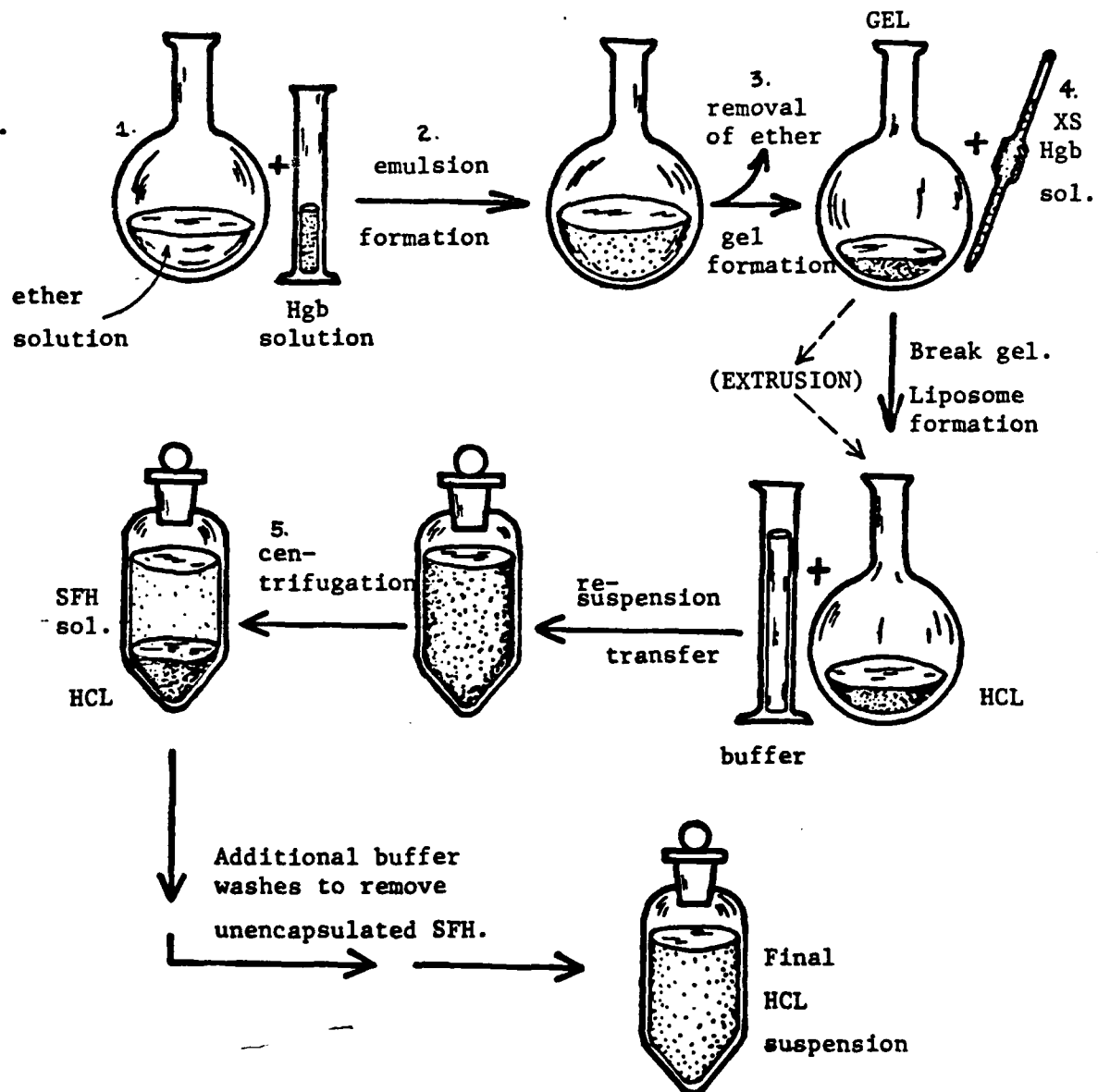




Figure 10. Front (upper) and side (lower) views of HCL in a dialysis cell. HCL, prepared by emulsion evaporation as described in the text, were placed in a two chamber dialysis cell to remove the unencapsulated SFH. The two chambers are separated by a 0.1μ Nucleopore membrane. The HCL remain in one chamber, the buffer in the second chamber is replaced frequently. In these pictures the unencapsulated SFH is about 90% removed.

Removal of Unencapsulated Hgb - We have evaluated three techniques to remove unencapsulated Hgb: (1) dialysis, (2) column chromatography, and (3) centrifugation and wash. All three procedures work but the last is the simplest.

We have used extrusion through Nucleopore membranes to prepare liposomes with refined size distributions (see F. Olson, C.A. Hunt et al., Biochem. Biophys. Acta, 557, 9, 1979 for more information). These membranes can be used in dialysis cells to retard liposomes smaller than the pore size used while allowing dialysis of untrapped Hgb. The buffer in the non-sample side (see Fig. 1a) is replaced every few hours until all free Hgb is removed from the sample side. We consistently do this at 4°. The procedure takes from 5 to 72 hrs; it is very rapid when a large, e.g. 1 μ , pore size is used but all liposomes significantly smaller than 1 μ , e.g. 0.5 μ , can eventually be dialyzed away along with untrapped Hgb. Using a smaller pore size, e.g. 0.1 μ , results in loss of few HCL but requires >24 hrs for complete removal of untrapped Hg.

Column chromatography does separate free from encapsulated Hgb as long as no HCL are larger than the spaces between the packed gel beads (this can be insured by extrusion as described in the above paper). The HCL are collected at the void volume of the column. The major disadvantages are that a 5-10 fold dilution of HCL results, and that many column runs are required to clean-up one large HCL preparation (there is a limit in the sample size that can be applied to the column while maintaining good separation).

The simplest and most rapid procedure is centrifugation and washing, similar to procedures used to isolate RBC's from blood. Because 7% SFH is more dense than isotonic buffer, once the HCL have been diluted slightly by buffer they can be centrifuged (we typically use 5-10,000 G for 10-20 min). The resulting supernatant is removed and the HCL pellet washed with 3 vols. (or more) of buffer. Repeating this process 4 times removes all unencapsulated SFH and the results are "cleaner" than those seen in Fig. 1a.

Separation of Hgb "Rich" from Hgb "Poor" HCL's - When washed HCL's are mixed 50/50 with various concentrations of aqueous sucrose (6 to 30%) and the resulting solutions are centrifuged in a hematocrit centrifuge, the liposomes are crudely separated based on density (Fig. 2). We have found that the most dense liposomes have the highest relative encapsulation of Hgb, i.e. highest Hgb to lipid ratio.

Figure 2 demonstrates two important points: 1) the Hgb-rich HCL can be separated from Hgb-poor HCL by selective density centrifugation; 2) these Hgb-HCL are relatively insensitive to hypertonic osmotic shock.

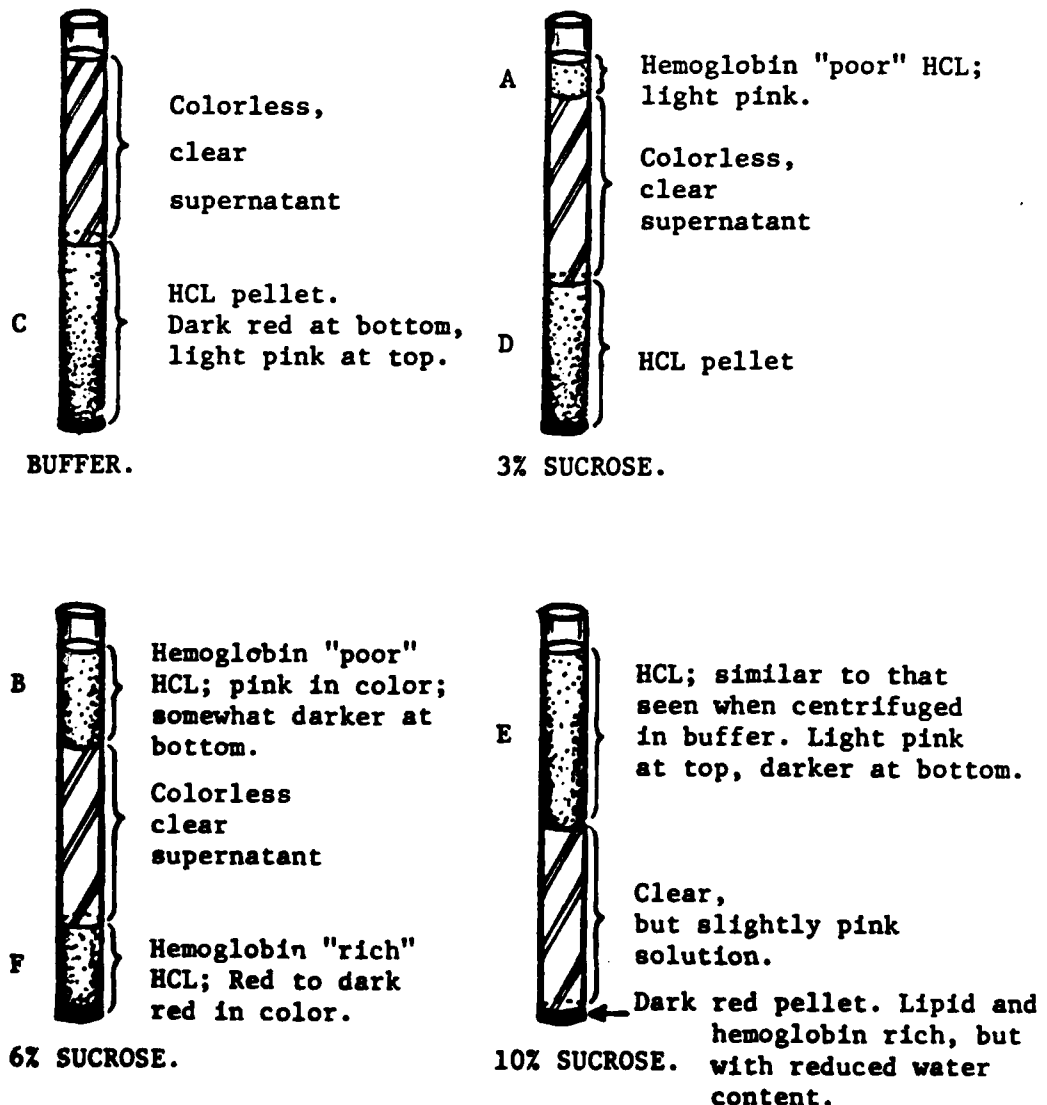
We have repeated the study described in Fig. 2 using ³H-PC in the lipid mixture and with ¹⁴C-sucrose added to the 7% Hgb. Following clean-up of HCL (removal of untrapped Hgb & ¹⁴C-sucrose) the ratio ³H/¹⁴C was measured in each separated phase. This ratio gives a measure of the relative amounts of trapped aqueous volume to lipid volume in each phase, and allows one to obtain a measure of the degree of leakage under each set of conditions.

The results are informative. The increase in the ³H/¹⁴C ratio parallels the decrease in red Hgb color seen following centrifugation (Fig. 2) suggesting that the very light-colored, Hgb-poor HCL, were probably multilamellar liposomes from which Hgb had been significantly excluded. The Hgb-rich HCL may be more unilamellar in nature. Only at sucrose concentrations 10% could freed sucrose be detected (less than 5% leakage) as a result of some hypotonic lysis.

FIGURE 2. Separation of hemoglobin "rich"

HCL (F) from hemoglobin "poor" HCL (A&B).

Aliquots of initial HCL suspensions are mixed with isotonic buffer or sucrose solutions to yield final sucrose concentrations of 0, 3, 6 or 10% followed by centrifugation in hematocrit tubes. The ratio of lipid (as ^3H) to trapped aqueous volume is $A > B > C \approx D \approx E > F$.



Oxygen Binding to HCL - Oxygen binding studies have not been a major focus of attention thus far for three reasons: (1) Preliminary evaluation of percent saturation vs pO_2 for HCL compared to 7% SFH indicated similarity suggesting that there was no major adverse effects of the Hgb being encapsulated. (2) It is difficult to accurately quantitate Hgb amounts in HCL suspensions. (3) The observation that the amount of Hgb in an HCL suspension, based on O_2 binding at 100% saturation, is approximately twice the Hgb level calculated using spectrophotometric techniques (Table 1; we are currently seeking an explanation for this observation).

Clearly, O_2 binding studies⁴ HCL should be carried out using radio-labeled (^{51}Cr :chromate) Hgb to avoid problems of Hgb quantitation. We have begun such studies. In the initial studies the specific activity of the labeled Hgb was insufficient for the task, but this can be improved. Details of this approach are provided in the renewal proposal.

Binding of SFH to Liposomes, We have evaluated SFH binding to liposomes with and without encapsulated Hgb using [^{51}Cr]chromate labeled Hgb and gel chromatography. In all studies the levels of radioactivity contained in the Hgb peak were not statistically different from background. Based on this data, at most 2% of the Hgb in our HCL could be bound on the outside of these liposomes (this is very likely an over estimate). However, it should be noted that "loose" binding of Hgb to these liposomes would not be detected by this method. Improved binding studies using Nucleopore membrane techniques are outlined in the Renewal Proposal.

RES Uptake of Liposomes. In our original proposal we indicated that a potential problem in the use of HCL as a blood substitute may be that such liposomes would be quickly taken up by tissues of the RES (this is a problem for FCE, also). We speculated that RES uptake of liposomes may not be the major mechanism for the blood clearance of liposomes. We have completed studies (discussed below) which support this hypothesis. Briefly, there are three important factors which govern the disposition and blood clearance of liposomes: (1) destabilization by plasma components, (2) binding to tissues and (3) RES uptake. Further, each of these factors or mechanisms is saturable. Thus it seems reasonable to speculate that the circulating life-time of HCL, at the high doses (numbers of liposomes) required of a resuscitative fluid will be dramatically longer than that seen for smaller, tracer doses of liposomes. Further, alterations in composition may decrease tissue binding and increase the circulating life-time even further.

If all liposomes are taken up by RE cells, then when ^{14}C -inulin is administered i.v. encapsulated in liposomes one should see a rapid rise in liver and spleen levels followed by a plateau. The fraction of the dose in vivo should be 100% of the dose at all times. Excretion of inulin would result only when liposomes become leaky or decompose before being taken up by cells. If liposomes are not maximally stable, then after some time any liposome which had not been taken-up by RE cells would have decomposed and the released inulin excreted. We have used these facts to evaluate RES uptake of liposomes as a function of dose. We used liposomes composed of PC/PA/CH/ α -T (4/1/1/.1), which have 'medium' stability properties in plasma. Doses of 1.5, 0.3 and 0.06 μM (total lipid) per g body weight were injected i.v. into mice, and various tissue levels plus carcass levels of ^{14}C -inulin were measured at selected times up to 72 hours. Extruded/cleaned-up 1.0 μ liposomes were used. Results are given in Figs. 3-5.

Using the relatively constant spleen levels as an indication, we conclude that the selected liposome composition was appropriate. We take the observed plateau levels

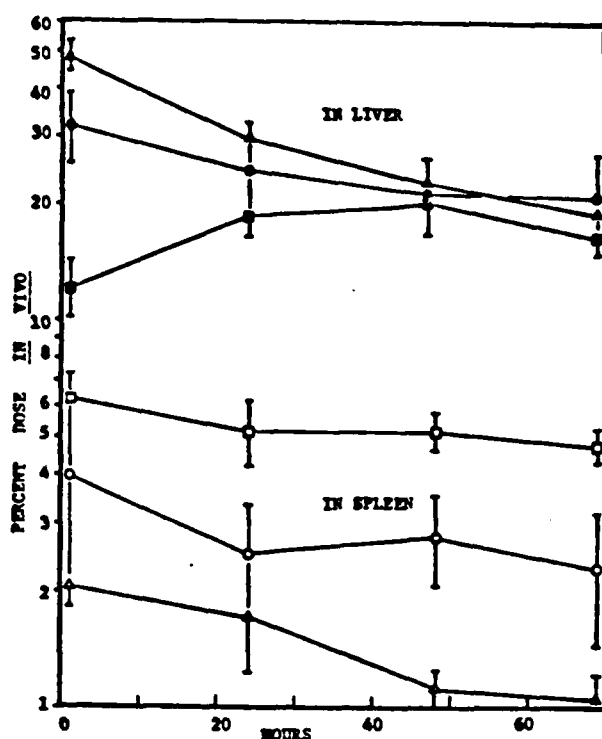


Figure 3. Percent of ^{14}C dose in livers and spleen of mice at selected times following i.v. injection of ^{14}C -inulin encapsulated in 1μ (diameter) liposomes composed of PC/PA/CH/ α -T (4/1/1/.1). The lipid doses per g body weight were $1.5\mu\text{M}$ (□,■), $0.3\mu\text{M}$ (○,●) and $0.06\mu\text{M}$ (Δ,▲). The vertical bars are one S.D. (n=3) or the range (n=2).

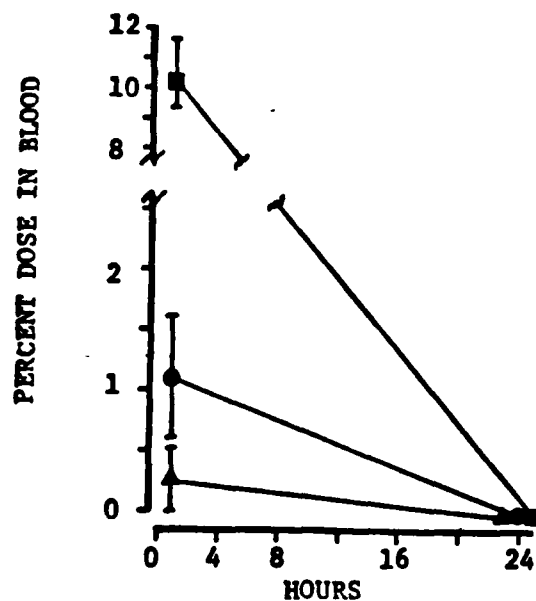


Figure 4. Blood levels in mice following high (■), median (●) and low (▲) doses of 1 liposomes. The study is the same as that in Fig. 3. Radio-activity levels were background at 24 hrs and later. The vertical bars are as described in Fig. 3.

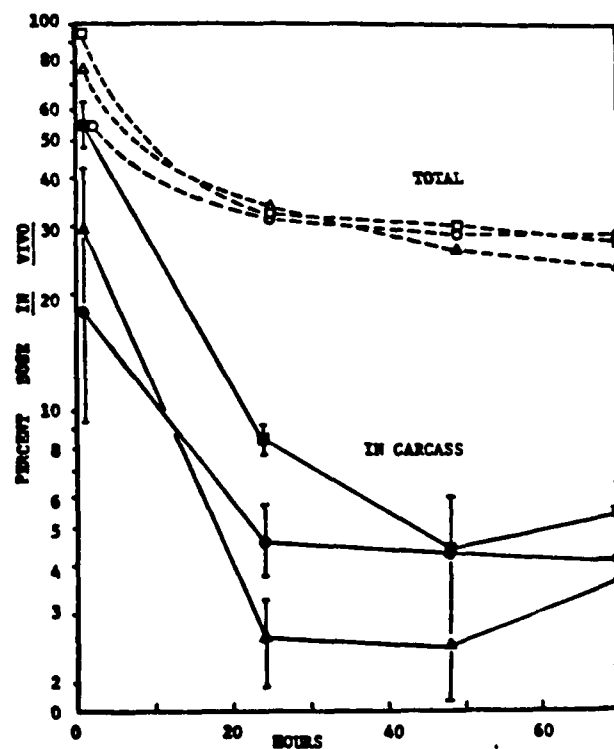


Figure 5. Percent dose in carcass and total in vivo for the study in Fig. 3. High (□,■), medium (○,●) and low (Δ,▲) doses. The vertical bars are as described in Fig. 3.

as an indication of the fraction of the dose taken up irreversibly by RE cells. We assume the remainder of the dose has been released from liposomes and excreted; this final stage is probably best indicated by various tissue levels at 70 hours.

We used 1 liposomes so that the amount of inulin per liposome would be sufficient to examine the tissue accumulation properties of a reasonably wide dose range. There is a modest dose effect. At the highest dose liver binding (Fig. 3) is apparently saturated as indicated by decreased liver and increased blood levels (Fig. 5).

The dose effect is only seen at early times in the liver, blood and carcass, but is seen at all times in the spleen (Figs. 3-5). A possible explanation is that liver uptake (as opposed to spleen uptake) was not saturated, whereas liver binding was. Further, uptake by the liver appears to be relatively slow. The total inulin remaining in vivo showed no dose effect (Fig. 5); apparently, in addition to liposome decomposition, the initial step of disposition is most likely binding of liposomes to all tissues followed by slow RES uptake. When liver binding is saturated, the excess liposomes either continue to circulate or bind to other tissues (not necessarily irreversibly). This spill-over results in an increase in spleen uptake (which is not large on a percent of dose basis) and association with other tissues (blood, carcass). With the continued redistribution of liposomes, liver-uptake continues until all liposomes either decompose or become essentially irreversibly associated with other tissues.

Reproducibility and Homogeneity of Liposome Preparations. We have developed extrusion techniques to prepare liposomes with an improved size distribution. Within the past few months we have improved on these procedures. Under normal dialysis conditions liposomes smaller than the pore size of a Nucleopore membrane will dialyze across the membrane in a first order fashion (Fig. 6). When the pore size is smaller than some fraction of the liposomes, the smaller liposomes can be dialyzed away (Fig. 7). We now use this technique to further improve the size distribution of extruded liposomes.

Daniel Friend, M.D. at this institution has allowed us access to his electron microscopy equipment on a recharge basis. We have constructed the size distributions of various liposome types using freeze fracture EM. Mechanically dispersed liposomes extruded through 1 membranes typically have size distributions as shown by the solid curve in Fig. 8 (frequency vs. observed diameter). The distributions are clearly not normal; however, the distribution of these extruded liposomes are approximately log-normal (Fig. 9).

A narrow and reproducible frequency-size distribution is important for evaluating the in vivo fate of liposomes as a function of size. However, from the standpoint of the encapsulated SFH, the volume of a liposome is more important than its diameter. When the total volume of 250 of the liposomes in Figs. 8 and 9 is calculated and the percent of total volume of the entire sample is plotted vs. diameter, a different, nearly normal, distribution is seen (Fig. 10). For this liposome sample the median size is 1/3 the median volume-adjusted size.

Reproducibility of liposome preparations has been a problem, but it need not be. Both extruded (using a 1 membrane) liposomes and extruded-dialyzed (using a 0.8 membrane) liposomes can be prepared reproducibly (Fig. 11). Figure 11 also shows how the dialysis technique improves and narrows the size distribution of extruded liposomes. Extrusion should be incorporated at an early stage in the preparation of HCL to insure an upper limit to HCL size.

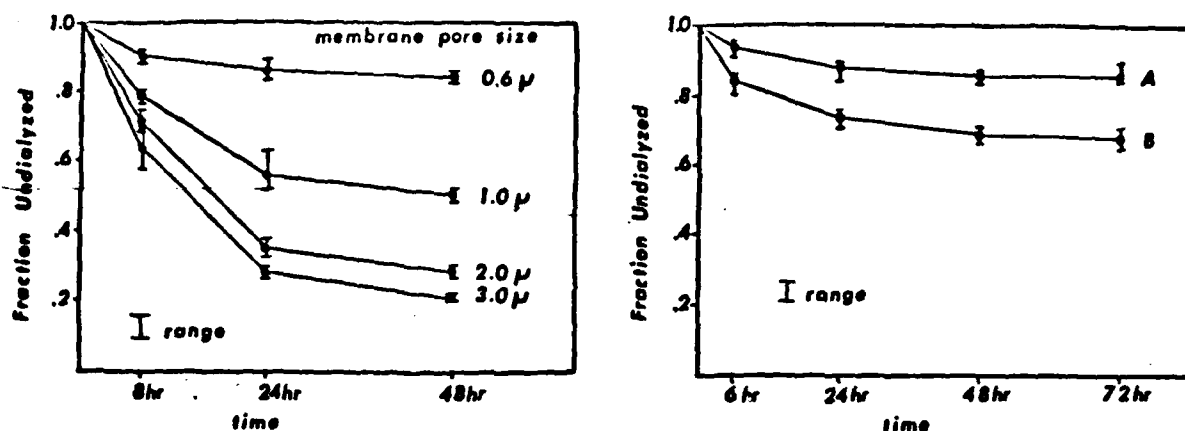


Figure 6. Dialysis of unextruded liposomes against nucleopore membranes of different size. Liposomes labelled with 14 C-cholesterol, CH. The 48 hr. values give an estimate of the percent larger than the membrane pore size.

B. Liposomes were extruded through 1μ membranes and then a fraction was extruded through a 0.4μ membrane. Curves shows results when an equal mixture of 1.0μ and 0.4μ extruded liposomes are dialyzed against 1 (A) and 0.4 (B) membranes. It should be noted that microspheres that are approximately 90% the diameter of the membrane pore do not dialyze across in 48 hours.

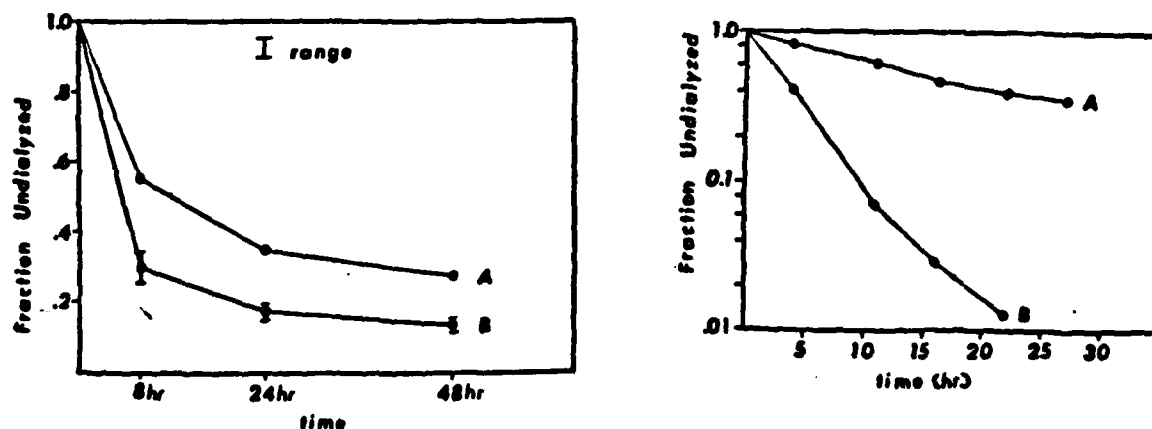


Figure 7. Dialysis of 1μ extruded liposomes against a 0.8μ membrane (Curve B). Curve A is for the same preparation that was pre-dialyzed for 20 hours against a 0.8μ membrane. B: Dialysis of small ($0.02-0.05\mu$) liposomes against a 0.1μ membrane (curve A) or against a 1μ membrane (Curve B).

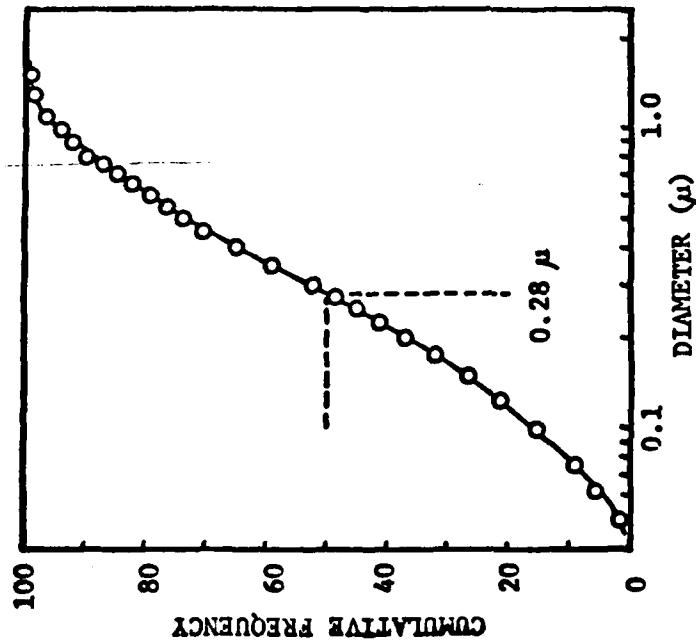


Figure 9. Frequency vs. log-diameter for liposomes extruded through a 1μ membrane (solid curve in Fig. 8). An 'S' shaped curve indicates an approximately normal distribution (log-normal).

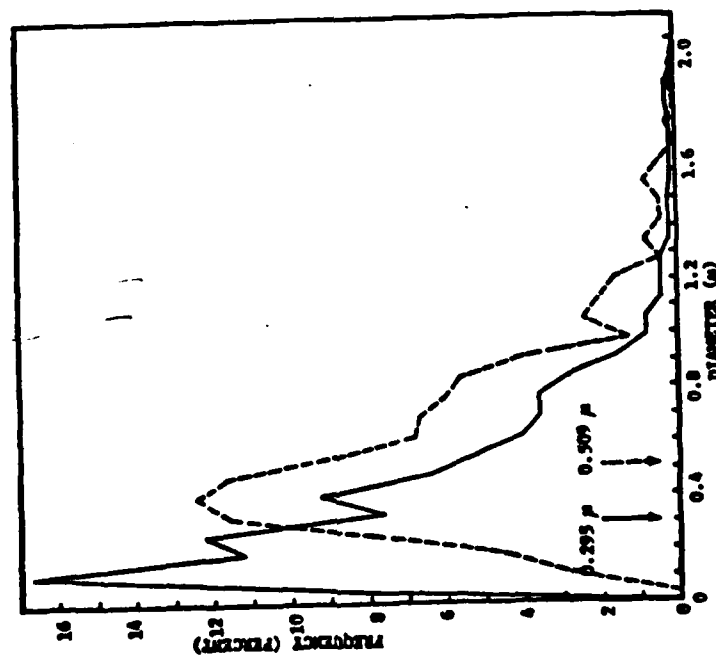


Figure 8. Frequency vs. diameter for liposomes extruded through a 1μ membrane. Solid curve: After extrusion without further clean-up. Dashed curves: after extrusion and 24 hours of dialysis against a 0.8μ membrane (which results in approximately 30% loss of lipid). Diameters are from freeze-fracture EM. Mean diameters are indicated by the arrows.

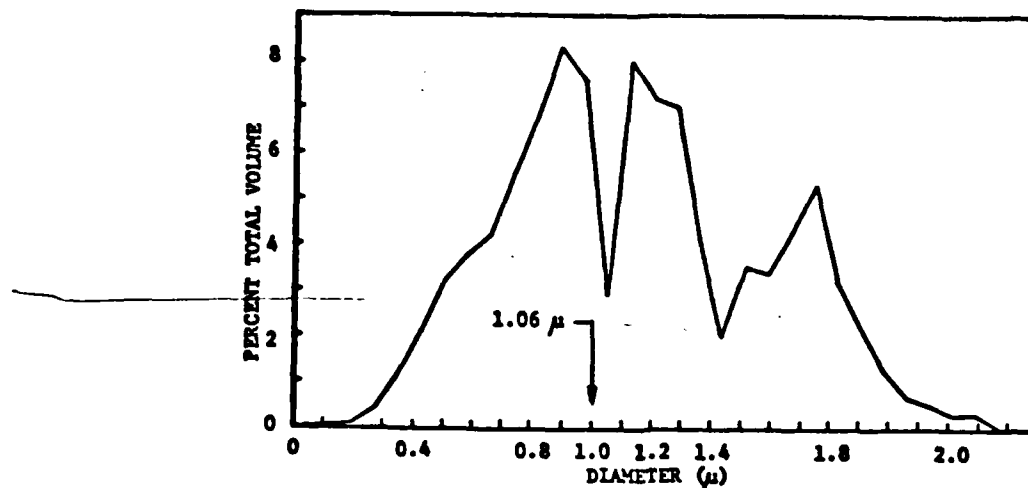


Figure 10. Frequency vs. volume-equivalent diameter. The total volume of the liposome sample described by the solid curve in Fig. 8 was calculated from the observed diameters assuming all liposomes were spheres. Percent total volume was then plotted vs. diameter. The mean volume-equivalent diameter is shown by the arrow.

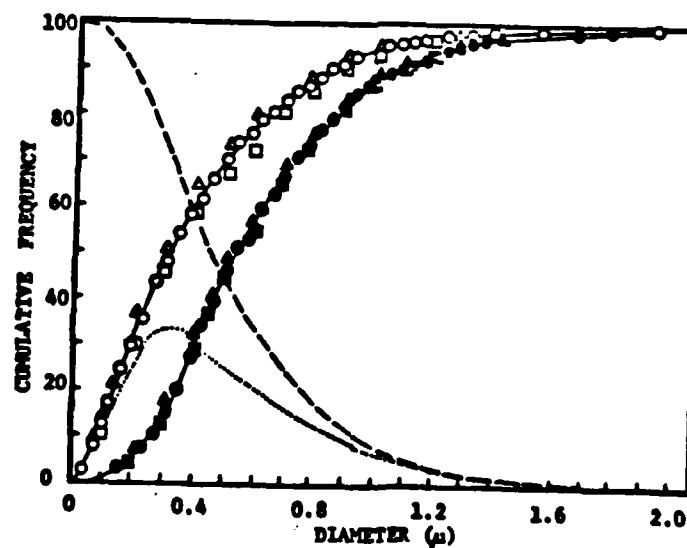


Figure 11. Cumulative frequency vs. diameter for the extruded, uncleaned (open symbols) and extruded, cleaned (closed symbols) liposomes similar to those shown in Fig. 8. The three different symbols are for three different batches. The solid curves give the percent of liposomes smaller than the indicated diameter. The dotted curve is a difference curve. The dashed curve is calculated from the area under the dotted curve; it represents the percent of the original sample that was dialyzed away.

Improving the Circulation Time of Liposomes. Clearly, tracer studies using a given HCL can not give a reliable estimate of the circulating half-life of the liposome since tissue binding, circulating stability and RES uptake are saturable. The available data suggests that at high doses, i.e. transfusion, the circulating life-time of liposomes will be dramatically longer than for tracer studies. However, it seems reasonable that tracer studies could be used to screen various liposome compositions; liposomes with increased circulation half-lives would be considered prime candidates for transfusion studies in the future.

We now have information (4) that liposomes composed of sphingomyelin/CH (ratio of 2/1) have a circulation half-life of 6 hrs and that 75% of the liposomes are in the circulation 1 hr postdosing. This data indicates that liposome composition studies must be carried out to identify the best candidate composition for HCL to be subsequently studied in animal transfusion studies.

Toxicity. Although we did not plan any toxicity studies (available data suggest that liposomes are not very toxic) we can report that there were no signs of acute toxicities from any of our in vivo work, even at the highest liposome doses.

Conclusions

Liposome-encapsulated stroma-free hemoglobin should be evaluated further as a potential blood substitute. This recommendation is based on the following observations.

1. Procedures have been developed to efficiently encapsulate SFH in liposomes.
2. The liposomes can be prepared reproducibly.
3. Encapsulation does not adversely affect oxygen binding of SFH.
4. Hemoglobin "rich" liposomes can be easily separated from hemoglobin "poor" liposomes.
5. The destabilizing effects of plasma, the tissue binding of liposomes and the RES uptake of liposomes are saturable at high liposome doses; at the high, transfusion, doses the circulation half-life of liposomes may be dramatically increased.
6. Changing composition can increase both the fraction circulating and the circulation half-life.
7. No signs of acute toxicity have been seen.

References

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